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Group 13 HOX Proteins Interact with the MH2 Domain of R-Smads and Modulate Smad Transcriptional Activation Functions Independent of HOX DNA Binding Capability

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Group 13 HOX proteins interact with the MH2 domain of R-Smads and modulate Smad transcriptional activation functions independent of HOX DNA-binding capability

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ABSTRACT

Interactions with co-factors provide a means by which HOX proteins exert specificity. To identify candidate protein interactors of HOXA13, we created and screened an E11.5–E12.5, distal limb bud yeast two-hybrid prey library. Among the interactors, we isolated the BMP-signaling effector Smad5, which interacted with the paralogous HOXD13 but not with HOXA11 or HOXA9, revealing unique interaction capabilities of the *AbdB*-like HOX proteins. Using deletion mutants, we determined that the MH2 domain of Smad5 is necessary for HOXA13 interaction. This is the first report demonstrating an interaction between HOX proteins and the MH2 domain of Smad proteins. HOXA13 and HOXD13 also bind to other BMP and TGF-β/Activin-regulated Smad proteins including Smad1 and Smad2, but not Smad4. Furthermore, HOXD13 could be co-immunoprecipitated with Smad1 from cells. Expression of HOXA13, HOXD13 or a HOXD13 homeodomain mutant (HOXD13$^{IQN>AAA}$) antagonized TGF-β-stimulated transcriptional activation of the pAdtrack-3TP-Lux reporter vector in Mv1Lu cells as well as the Smad3/Smad4-activated pTRS$_6$-E1b promoter in Hep3B cells. Finally, using mammalian one-hybrid assay, we show that transcriptional activation by a GAL4/Smad3-C-terminus fusion protein is specifically inhibited by HOXA13. Our results identify a new co-factor for HOX group 13 proteins and suggest that HOX proteins may modulate Smad-mediated transcriptional activity through protein–protein interactions without the requirement for HOX monomeric DNA-binding capability.

INTRODUCTION

Mammals have 39 *Hox* genes that encode transcription factors that are essential for normal development (1). Mutations in group 13 *HOX* genes have been described in the human *HOXA13* and *HOXD13* genes, and engineered and spontaneous mutants of both genes have added significantly to our understanding of their role in development (2–15). However, our knowledge of how these proteins gain specificity of function in different tissues is limited.

One mechanism whereby HOX proteins achieve functional specificity is by interaction with additional DNA-binding co-factors, of which PBX and MEIS proteins to date are the most studied (16). PBX proteins interact cooperatively on DNA with HOX proteins of paralog groups 1–10 but not groups 11–13 (17,18), while MEIS proteins cooperatively bind to DNA with paralog groups 9–13 (19). Interactions have also been demonstrated to occur between MEIS proteins and HOXA13 and HOXD13, as well as non-*AbdB*-like HOX proteins in the absence of DNA (20). However, in the developing limb bud mesenchyme *Meis1-3* are expressed in a separate more proximal region than the distally restricted expression of *Hoxa13* and *Hoxd13* (20–22), thus precluding a productive interaction.

We sought to identify potential co-factors of HOXA13 using a yeast two-hybrid screen of a developmentally appropriate limb bud cDNA library. Among the isolated candidates, we found Smad5, a well-described mediator of BMP function (23,24) and a critical gene for embryonic and extra-embryonic vascular development (25,26). Owing to the importance of

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

Hoxa13 in limb, genitourinary tract, umbilical artery and digestive tract development, we chose to analyze HOXA13/Smad interactions and function in detail.

MATERIALS AND METHODS

Limb bud yeast two-hybrid cDNA prey library

Forelimb and hindlimb bud autopods of 140 C57BL/6J embryos were harvested between E11.5 and E12.5, stages 4–10 (27). Total cellular RNA was purified using Trizol reagent (Invitrogen Corporation). From 886 μg of total autopod RNA, 5.9 μg of poly(A) RNA was obtained using the Oligotex mRNA midi kit (Qiagen). Random-primed cDNAs were created using the SuperScript Choice System (Invitrogen Corporation) with EcoRI adapters and were ligated into the EcoRI site of the prey vector pJG4-5 (Origene Technologies). DNA from ligations was transformed into electrocompetent DH10B cells (Invitrogen Corporation) producing 1.8 million insert containing colony forming units (CFUs). Prey vector DNA from pooled colonies was transformed into the haploid yeast EGY188 (MATa) following the DupLEX-A large-scale library transformation protocol. A total of 85 transformations, each using 1 μg of DNA, were performed. Transformants were selected on YNB (glucose) medium lacking tryptophan (trp). Aliquots containing 3.6×10^6 yeast CFUs were pooled for use in screening. Using dilution plating onto YNB (glucose) medium lacking tryptophan, we estimated the pooled library titer to be 6.6×10^8 CFU/100 μl.

Two-hybrid library screening

A HOXA13 bait construct was created by cloning the coding sequence for amino acids 150–360 into the vector pEG202NLS, placing the coding sequence 3′ of the LexA DNA-binding domain (DBD) and nuclear localization signal (NLS). Bait plasmids and the lacZ reporter vector (pSH18-34) were transformed into the haploid yeast strain EGY40 (MATα) and transformants were selected for growth on YNB (glucose) medium lacking histidine (his) and uracil (ura). The limb bud prey library was screened using interaction mating (28,29). Aliquots containing 3×10^8 CFUs were mated to the opposite mating type and diploid interactors were selected for growth on YNB (galactose) -his -ura -trp -leu medium. Bait autoactivation potential was calculated as the ratio of colonies growing on YNB (galactose) -his -ura -trp -leu versus -his -ura -trp (+leu). The ratio was used to determine the number of interactors to be screened based on the product of the bait autoactivation potential and the number of unique preys in the prey library (28). The calculated CFU from the bait/prey library mating was replica plated on both YNB (galactose) -his -ura -trp +X-gal and YNB (glucose) -his -ura -trp +X-gal medium. Those turning blue only on the galactose containing plates (prey protein expressed) were characterized further. Prey plasmids were isolated from diploid yeast following the DupLEX-A protocol and sequenced using the 5′ target fusion primer: 5′-CTGAGTGGAGATGCCTCC-3′. Candidate identification was performed using BlastN analysis of insert DNA sequence and BlastP analysis of open reading frames (30).

Additional yeast two-hybrid preys were created by cloning selected coding sequences into the prey vector pJG4-5, on the 3′ end of the B42 transcriptional activation domain (AD), and bait constructs were made by cloning coding sequences 3′ of the LexA DBD of the vectors pEG202 or pEG202NLS (includes NLS). The specificity of interaction was assessed for Smad5 and other preys by transformation of haploid yeast strain EGY48 (MATα) followed by interaction mating. Constructed bait vectors, pRFHMI (Bicoid HD), pBait (TGFβRI), pSH17-4 (LexA DBD-GAL4 AD), pEG202 (LexA DBD) and pEG202NLS (LexA DBD-NLS) were each cotransformed into the haploid yeast strain RFY206 (MATa) with lacZ reporter vector (pSH17-4). Interaction mating and visual scoring was performed 24 h after growth at 30°C on glucose and galactose medium (-trp, -his, -ura) with X-gal.

Sequence analysis

Accession numbers for the *Mus musculus* protein sequences: HOXA13 (NP_032290), HOXD13 (NP_032301), Smad1 (AAG41407), Smad2 (AAH21342), Smad3 (NP_058049), Smad4 (NP_032566), Smad5 (NP_032567), Smad6 (NP_032568), Smad7 (CAA04182) and Smad8 (AAF77079). The accession numbers for *Drosophila melanogaster* are Mad (AAB60230) and Medea (AAC38971). Comparison of protein sequences was carried out using the MEGALIGN program (DNASTAR) with the CLUSTAL W alignment algorithm (31). A neighbor-joining tree was generated in MEGALIGN, from the CLUSTAL W sequence alignments of full-length protein sequences.

Plasmids

pAdtrack-3TP-Lux (32), $pTRS_6$-E1b-luc (33,34) and pG5B-luc (33) luciferase reporter plasmids have been described previously. Briefly, 3TP-Lux is a chimeric reporter that contains promoter elements of the human collagenase and PAI-1 genes (35). This reporter is activated by TGF-β stimulation in a variety of cells. The $pTRS_6$-E1b-luc promoter is composed of six copies of the −732/−721 Smad3/Smad4 binding segment of the human PAI-1 promoter upstream of the E1bTATA box in pE1b-luc (34). pG5B-luc has five copies of the GAL4 DNA-binding site upstream of the E1bTATA box in pE1b-luc (33). Transfection controls included either *Renilla* luciferase or chicken β-actin-promoter-β-galactosidase. pCMV5-*Hoxa13*, pCMV5-*Hoxd13*, pCMV5-*Hoxd13*$^{IQN>AAA}$, pCMV5-*Hoxa9* and pCMV5-*Hoxd11* encode full-length cDNAs for each mouse gene. Negative control plasmids are pCMV5 (empty vector), pPAI-RBP1 [pCMV backbone driving plasminogen activator inhibitor RNA-binding protein 1 (36)] and p37-AUF1 (a pCMV vector driving expression of AUF RNA-binding protein) (a kind gift from Robert Schneider). GAL4-Smad3C has been described previously (33) and expresses a GAL4 DBD fusion protein composed of amino acids 172–425 (the C-terminal activation domain) of human Smad3. GAL4-VP16 is the GAL4 DBD with a VP16 AD (33). N-terminal FLAG-epitope-tagged Smad1 expression vector was used in conjunction with constitutively activated BMP type IA receptor (ALK3) vector for Mv1Lu cell assays with the 3TP-Lux reporter and these have been described previously (37,38).

Co-immunoprecipitation

The day prior to transfection 325 000 C3H 10T1/2 cells were seeded into 60 mm dishes (3 per transfection condition). Prior

to transfection, cells were washed with phosphate-buffered saline (PBS) and incubated with media lacking antibiotics. Transfections were performed using the FuGENE 6 reagent (Roche Applied Science). Cells were transfected with expression constructs as indicated in Figure 4. Aliquots containing 2 μg each for pCMV5-*Hoxd13* and FLAG-Smad1 (37,38) expression vectors and 200 ng of the constitutively activated BMP type IA receptor (ALK3) were used for transfection. Forty-eight hours post-transfection, cell lysates were prepared by collecting cells from three 60 mm dishes with trypsin-EDTA. For each condition, cells were pelleted and lysed in 1 ml cold RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 1% NP40, 0.5% DOC and 0.1% SDS). Mechanical disruption with a 21G needle and brief sonication were used to ensure efficient disruption of nuclei. Cellular debris was pelleted by centrifugation at 10 000 *g* for 10 min and lysates were used for IP with anti-FLAG M2 affinity gel following the manufacturer's protocol (Sigma–Aldrich). Proteins were separated by electrophoresis on 12% SDS–polyacrylamide gel and transferred to nitrocellulose for western analysis. We used primary antibody dilutions of 1:10 000 for rabbit anti-HOXD13, 1:1000 for rabbit anti-Smad1 (Upstate Biotechnology), 1:1000 for mouse M2 anti-FLAG (Stratagene) and 1:10 000 each for anti-rabbit and mouse horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) in PBST with 5% Carnation nonfat dry milk. Proteins were visualized using Supersignal chemiluminescent substrate (Pierce Biotechnology).

Cell culture, transfection and luciferase assays

C3H 10T1/2 (mouse embryonic fibroblasts), mink Mv1Lu cells (32) and Hep3B (human hepatoma) cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Invitrogen Corporation). All transfections were performed using FuGENE 6 in 6- or 12-well plates. For Mv1Lu cells, the day prior to transfection 150 000 cells were seeded into individual wells of 6-well plates and then transfected with 25 ng of *Renilla* luciferase normalization vector pRLSv40, 1 μg of the luciferase reporter vector pAdtrack-3TP-Lux and 2 μg of either pCMV5, pCMV5-*Hoxa13*, pCMV5-*Hoxd13* or pCMV5-*Hoxd13*$^{IQN>AAA}$. Twenty-four hours post-transfection, cells were washed with PBS and then grown for an additional 24 h in serum starvation medium (SSM) with either TGF-β1 or vehicle. SSM consisted of DMEM with 0.2% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. TGF-β stimulation was performed using SSM supplemented with recombinant human TGF-β1 (R & D Systems) at 100 pM or vehicle (32,39). For these 3TP-Lux experiments, *Renilla* luciferase was used as the transfection control. For experiments with pTRS$_6$-E1b-luc or in mammalian one-hybrid assays with pG5B-luc in Hep3B cells chicken β-actin-promoter-β-galactosidase activity was measured (Clontech) as the transfection control. Sixteen hours after transfection, the cells were treated with 50 pM TGF-β1 for 24 h. In control experiments, dexamethasone inhibited TGF-β stimulation of pTRS$_6$-E1b-luc expression in Hep3B cells as expected (33) (data not shown). Cell lysates were prepared for measurement of luciferase activity according to the manufacturer's protocol (Promega Corporation) and readings were taken using a Monolight 3010 (BD Biosciences PharMingen). All luciferase readings were normalized to the transfection control readings. The average of several experiments each performed in triplicate with 1 SD is presented as a percent of control TGF-β stimulation over basal activity. Duplicate transfected samples were analyzed by western blotting to verify and compare the expression of HOX proteins (data not shown).

RESULTS

Construction and characterization of a mouse limb bud prey library

We created a random-primed yeast two-hybrid prey library from E11.5–12.5 C57BL/6J forelimb and hindlimb buds, a tissue and time during development known to exhibit strong *Hoxa13* expression. A total of 1.8 million independent cDNA inserts were obtained in the yeast two-hybrid prey vector pJG4-5. We predicted that one-sixth of the inserts (300 000), on average, would be in the correct orientation and reading frame. Insert-spanning PCR of 50 random clones showed the average insert length to be 824 bp with a range between 100 and 1800 bp.

HOXA13 specifically interacts with Smad5

To identify candidate HOXA13-interacting proteins, we used a bait molecule comprising murine HOXA13 amino acids 150–360 (full-length protein is 1–386) fused in-frame on the C-terminus of the LexA DBD with an NLS. This bait has been utilized elsewhere to study HOXA13 co-factor interactions (20). An estimate was derived for the number of colonies necessary to be analyzed to identify potential interactions with molecules of low abundance in the limb bud prey library. The preys from 162 colonies that could grow on leucine-deficient medium and were positive for lacZ activity were isolated and sequenced. BlastN and BlastP were used to identify genes and to determine whether the isolated preys represented coding sequences in the correct reading frame. Of those, 56 preys were reintroduced into yeast and 13 were found to interact specifically with the HOXA13 150–360 bait and not with the negative control baits encoding the Bicoid homeodomain (HD), TGFβRI or the LexA DBD-NLS alone (data not shown). In this paper, we present our data with one of these specific interactors, the BMP-signaling effector Smad5, and explore further the HOX/Smad range of interactions, peptide domains and function.

The Smad5 prey isolated from the library encoded amino acids 175 through 465 (full-length = 1–465). This clone represented a large portion of the linker and the MH2 domain of Smad5. We inserted this Smad5 coding sequence (175–465) into the bait vector, pEG202, and tested for interaction with a HOXA13 prey encoding amino acids 150–360. Reversal of the bait/prey orientation did not alter the specific interactions observed between HOXA13 and Smad5 (data not shown).

Interaction between Smad5 and paralog group 13 HOX proteins is not a general property of all *AbdB*-like HOX proteins

To test whether paralogous and non-paralogous HOX proteins could interact with Smad5, we created preys including amino

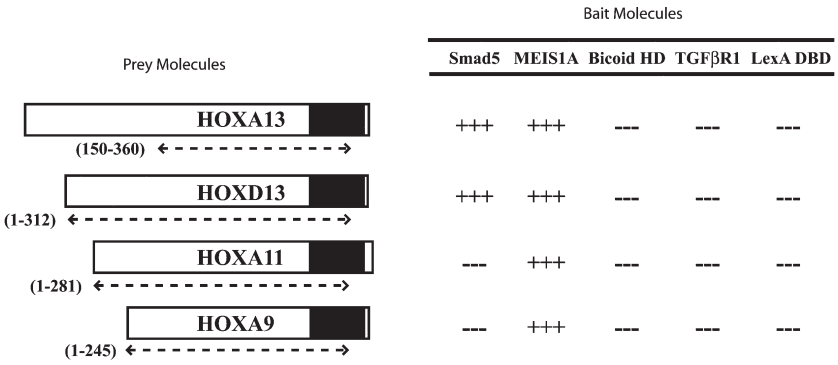


Figure 1. HOXA13 and HOXD13 interact specifically with Smad5. Bait proteins were tested for interaction with the HOX paralog group 13 proteins, HOXA13 (amino acids 150–360) and HOXD13 (amino acids 1–312) and with HOXA11 (amino acids 1–281) or HOXA9 (amino acids 1–245). A bait containing full-length MEIS1A, a known co-factor of these HOX proteins, interacted with each HOX prey tested. Interactions do not occur with the control bait proteins, including the LexA DBD alone, the Bicoid homeodomain (HD) or TGFβR1, showing that the interactions with HOXA13 and HOXD13 are specific. Positive interactions are reported as '+++' and no observed interaction as '−−−'.

acids 1–312 of mouse HOXD13, 1–281 of mouse HOXA11 and 1–245 of mouse HOXA9 (Figure 1) and tested them by interaction mating with a bait containing amino acids 175–465 of Smad5. All HOX preys terminate at homeodomain residue 41 (41/60), similar to that of HOXA13 (150–360). All tested HOX preys were shown to interact with a positive control bait containing MEIS1A (20); however, only the group 13 paralogs could interact with Smad5 amino acids 175–465 (Figure 1). The overall specificity of this interaction was further supported by the inability of any of the HOX preys to interact with either the Bicoid (HD), TGFβR1 or the LexA DBD alone baits. Group 10 and 12 HOX proteins were not tested, but the data indicate that the interaction is not a general property of all AbdB-like proteins.

HOXA13 interacts with the MH2 domain of Smad5

Previous studies showing binding between HOXC8 and Smad1 identified two HOX-interacting domains, HID1 and HID2, in Smad1 that were necessary for the interaction (40,41). Our isolated Smad5 prey did not retain the homologous HID1 region and was truncated within the homologous HID2 region, suggesting the possibility that HOXA13 interaction was mediated through different Smad5 amino acids. To identify the Smad5 domain(s) mediating HOXA13 interactions, additional prey constructs were tested (Figure 2). Smad5 preys with amino acids 1–198 (includes conserved MH1 domain, and both homologous HID1 and HID2 motifs), 146–198 (HID2 homologous region only) or 1–265, which includes the MH1 domain, HID1 and HID2 homologous domains and the adjacent C-terminal linker regions, failed to interact with HOXA13. However, preys including Smad5 amino acids 146–465, 202–465 and 265–465, all of which include the MH2 domain, could interact. These results demonstrate that the C-terminal amino acids of Smad5 that include the MH2 domain are sufficient for HOXA13 interaction.

HOXA13 and HOXD13 interact with the MH2 domain of other BMP and TGF-β/Activin-regulated Smad proteins

BMP ligands stimulate their cognate receptors to activate the receptor-regulated Smads (R-Smads) Smad1, Smad5 and

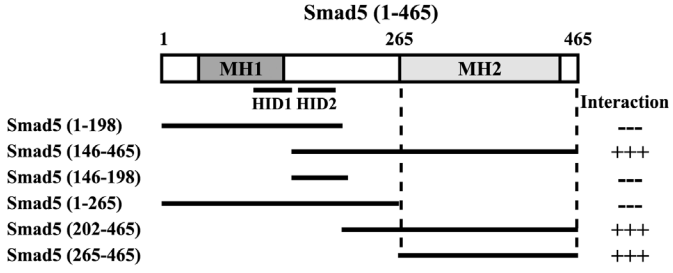


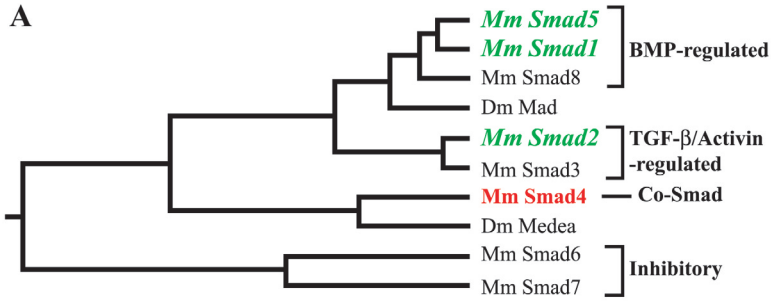
Figure 2. HOXA13 interacts with the C-terminal MH2 domain of Smad5. Interaction was tested between a bait molecule including HOXA13 amino acids 150–360 and prey molecules containing truncated Smad5 peptides. HOXA13 specifically interacted with all tested Smad5 preys containing the C-terminal MH2 domain (146–465, 202–465 and 265–465). Preys without the MH2 domain failed to interact.

Smad8 (23,24,42). These activated R-Smads dimerize in the cytoplasm with the common Smad, Smad4, and subsequently translocate to the nucleus where they participate in the regulation of gene expression in combination with other transcriptional regulators. To determine whether other Smad proteins are capable of interacting with HOXA13 and HOXD13, prey vectors were created corresponding to the C-terminal MH2 domain regions for the BMP effector Smad1, TGF-β/Activin effector Smad2, and the common dimerization partner of all BMP and TGF-β/Activin regulated Smads, Smad4. Interactions of comparable intensity were observed between the paralog group 13 HOX proteins and both Smad1 and Smad2, but not with Smad4 (Figure 3A). Interactions were not tested for the inhibitory Smads (I-Smads), Smad6 and Smad7, as their amino acid sequence identity in the respective C-terminal MH2-containing domains is markedly lower (Figure 3B). These experiments demonstrate that HOXA13 and HOXD13 are able to interact in yeast with receptor-regulated Smad proteins downstream of both TGF-β/Activin and BMP pathways, but not with the common Smad, Smad4.

HOXD13 and Smad1 interact in cells

To determine whether full-length Smad proteins and a paralog group 13 HOX protein are capable of associating in the context

A



B

	% Identity to Smad5 MH2	HOXA13/D13 Interaction
Mm Smad5 (265-465)	-----	Yes
Mm Smad1 (265-465)	97.5	Yes
Mm Smad8 (228-428)	90.5	ND
Dm Mad (255-455)	86.6	ND
Mm Smad2 (268-467)	78.6	Yes
Mm Smad3 (226-425)	78.1	ND
Mm Smad4 (316-551)	46.3	No
Dm Medea (544-771)	44.3	ND
Mm Smad6 (327-496)	30.3	ND
Mm Smad7 (255-426)	26.9	ND

Figure 3. Paralog group 13 HOX proteins interact with MH2 domains of BMP and TGF-β/Activin-regulated Smad proteins. (**A**) Neighbor-joining tree generated by CLUSTAL W for *Mus musculus* Smad proteins and the *Drosophila* orthologs Mad and Medea. Those demonstrated to interact with HOXA13 (amino acids 150–360) are shown in green italics, no interaction (Smad4) in red and not tested (no color). (**B**) Amino acid identity to the Smad5 C-terminal region, containing the MH2 domain and ability to interact with HOXA13 and HOXD13. ND, interaction potential was not determined.

of mammalian cells, co-immunoprecipitation was performed (Figure 4). C3H 10T1/2 embryonic fibroblast cells were transfected with expression vectors for the constitutively activated BMP receptor ALK3 and HOXD13 with or without co-expression of N-terminal FLAG-epitope-tagged Smad1. Using anti-FLAG antibody, HOXD13 was recovered only in co-immunoprecipitations when FLAG-Smad1 was co-expressed (Figure 4, lane 6). These results demonstrate association *in vivo* of a paralog group 13 HOX protein and receptor-regulated Smads, consistent with the yeast two-hybrid results.

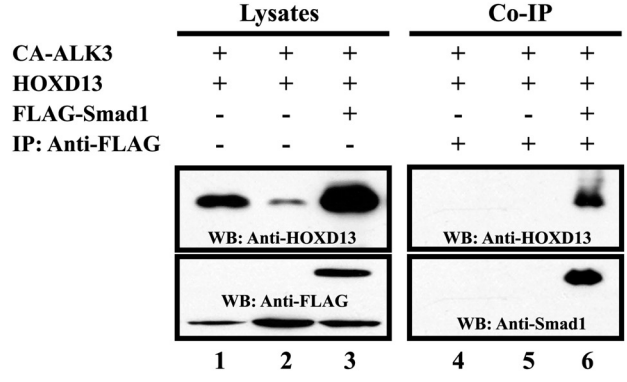


Figure 4. HOXD13 and Smad1 interact in mammalian cells. Co-immunoprecipitation demonstrates an *in vivo* interaction between both full-length Smad1 and HOXD13. The constitutively active (CA) BMP type IA receptor (ALK3) and HOXD13 were expressed in C3H 10T1/2 cells with or without co-expression of FLAG-Smad1. Whole cell lysates were prepared and subjected to immunoprecipitation using an anti-FLAG agarose conjugate. Cell lysates (lanes 1–3) and proteins eluted from the agarose conjugate (lanes 4–6) were resolved by SDS–PAGE and subjected to western blotting separately with anti-HOXD13, anti-FLAG and anti-Smad1 antibodies. Lanes 3 and 6 are from cells transfected with CA-ALK3, HOXD13 and FLAG-Smad1 expression constructs, and lanes 1, 2, 4 and 5 from cells transfected with CA-ALK3 and HOXD13 expression constructs. HOXD13 co-immunoprecipitated with FLAG-Smad1 in the absence of co-transfected CA-ALK3 as well (data not shown).

HOX group 13 proteins antagonize TGF-β1-stimulated transcriptional activation

To determine whether group 13 HOX expression has a molecular effect on Smad-mediated signal transduction, we utilized the 3TP-Lux chimeric reporter that contains upstream Smad binding elements from the human collagenase and PAI-1 genes (35). This reporter has been demonstrated to be activated by TGF-β stimulation in cells, including Mv1Lu, through Smads (32,37,39,43,44) and has been useful to demonstrate the consequences of co-expression of Smad-interacting proteins on reporter activity (45). Here, we expressed HOXA13, HOXD13 or HOXD13$^{IQN>AAA}$ in cells along with the 3TP-Lux reporter (Figure 5). Luciferase activity measurements were taken for cells treated with vehicle or TGF-β1 and compared with cells transfected with empty vector. Comparable levels of protein expression of HOXA13, HOXD13 and HOXD13$^{IQN>AAA}$ were observed in duplicate transfections via western analysis comparing equal amounts of cellular protein (data not shown).

TGF-β1 stimulated 3TP-Lux reporter expression an average of 6.3-fold (Figure 5), which is comparable with previous

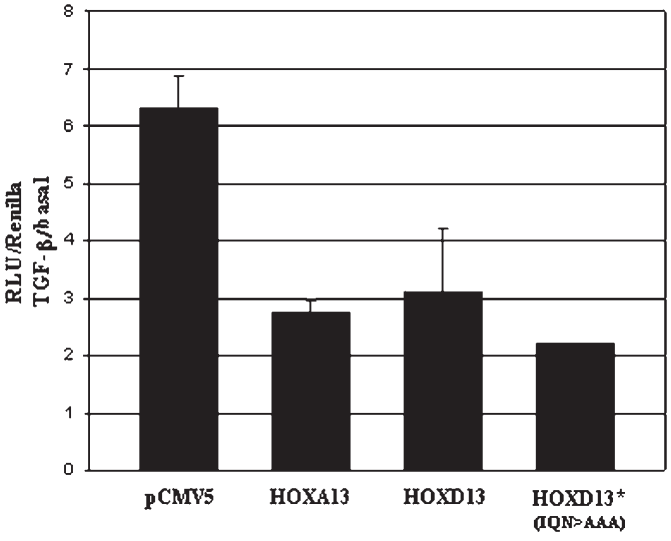


Figure 5. HOX group 13 proteins antagonize TGF-β-stimulated 3TP-Lux reporter activation. The effect of paralog group 13 HOX protein expression on fold stimulation provided by TGF-β1 stimulation was compared with empty vector control. Luciferase expression was consistently repressed in cells expressing HOXA13, HOXD13 or the DNA-binding mutant HOXD13 ($HOXD13^{IQN>AAA}$). Asterisk indicates that the experiment was only performed once in duplicate.

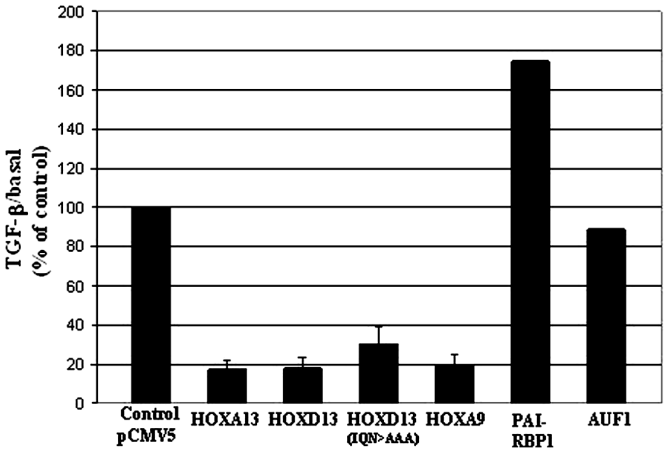


Figure 6. HOX proteins repress Smad-mediated transcriptional activation. Hep3B cells in 6- or 12-well plates were co-transfected with TRS_6E1b-luc reporter construct (500 ng/well of 12-well plate) and control (pCMV5), HOX, or negative control construct as indicated (500 ng). *Renilla* luciferase (4 ng) or β-galactosidase (40 ng) vector was included to correct for transfection efficiency. Sixteen hours after transfection TGF-β (50 pM) or vehicle was added and incubation continued for 24 h. Cells were harvested in cell lysis buffer (Promega) and assayed for luciferase activity. For each sample, luciferase activity was normalized to the activity of transfection controls and fold stimulation by TGF-β determined. TGF-β induction in control was set at 100% and induction in the presence of each test plasmid is expressed as a percentage of that in control. Triplicate samples were done in each experiment. The average TGF-β induction from four experiments was 14-fold. Data are taken from 2–4 experiments for HOX constructs and a single experiment for the negative controls.

reports showing 8- and 6-fold, respectively (32,39). Co-expression of HOXA13 and HOXD13 reproducibly repressed reporter activation (down from 6.3-fold to 2.75- and 3.1-fold, respectively). To determine whether HOX monomeric DNA-binding capability is necessary for repression, we analyzed the effect of the HOXD13 DNA-binding mutant protein $HOXD13^{IQN>AAA}$ (46,47). In this protein, the homeodomain residues I^{47}, Q^{50} and N^{51} are converted to alanine residues. This mutant protein was able to comparably repress TGF-β1-stimulated activation of the reporter (down to 2.2-fold). This work shows that paralog group 13 HOX proteins antagonize TGF-β1-stimulated gene expression, presumably through interaction with endogenous Smads, and this effect does not require HOX DNA-binding. In addition, we can conclude that the repression of luciferase reporter expression is not likely through binding of HOX proteins to the 3TP-Lux promoter or vector DNA.

HOX group 13 proteins antagonize Smad-mediated transcriptional activation

We next wanted to test whether HOX group 13 proteins could interfere with Smad-mediated transcriptional activation of the $pTRS_6$-E1b-luc reporter and to show conclusively in another promoter reporter whether monomeric DNA-binding capability by HOX group 13 proteins is critical for downregulation of Smad-mediated transcriptional activation. The $pTRS_6$-E1b promoter is composed of six copies of the −732/−721 segment of the human PAI-1 promoter that has been shown to bind a TGF-β1-inducible protein complex, including Smad3 and Smad4 (34) upstream of the E1bTATA box in pE1b-luc. The MH1 domain of Smad3 and full-length Smad4 bind the 12 bp TRS element, which is necessary and sufficient for Smad binding and for TGF-β1-stimulated expression. In co-transfected Hep3B cells HOXA13, HOXD13 and $HOXD13^{IQN>AAA}$ reduced TGF-β1-stimulated expression 5-fold, whereas the negative controls (PAI-RBP1 and AUF1) did not affect the expression (Figure 6). HOXA9 was tested for the ability to repress stimulation since it is known to bind to Smad4, but not Smad3 (48). HOXA9 also reduced Smad-mediated transcriptional activation, suggesting that not only can Smad4 bind to HOXA9 and relieve HOXA9 DNA binding and transcriptional repression (48), but HOXA9 can interfere with Smad-mediated activation of transcription.

HOX group 13 proteins antagonize Smad3-MH2 domain-mediated transcriptional activation

The inhibitory effect of HOX group 13 proteins on Smad-mediated activation of pTRS6-E1b-luc expression could occur through several mechanisms and is complicated by the interactions that occur between the MH1 and MH2 domains of Smads (49). To test whether HOX group 13 proteins could inhibit activation functions of the Smad3 MH2 domain, we used a mammalian one-hybrid approach. GAL4-Smad3C protein, which substitutes the GAL4 DBD for the MH1 domain of Smad3, reproducibly stimulated expression on a GAL4 luciferase reporter, pG5B (Figure 7, left). Co-transfection with HOXA13, by comparison with the pCMV5 empty vector, reduces the expression 5-fold. Similar repression is observed despite incubation with TGF-β1 (data not shown). Activation was also reduced 2.4-fold by HOXD13 and $HOXD13^{IQN>AAA}$, but was unaffected by PAI-RBP1 (data not shown). HOXA13 did not repress the activation of pG5B expression by GAL4-VP16 (Figure 7, right).

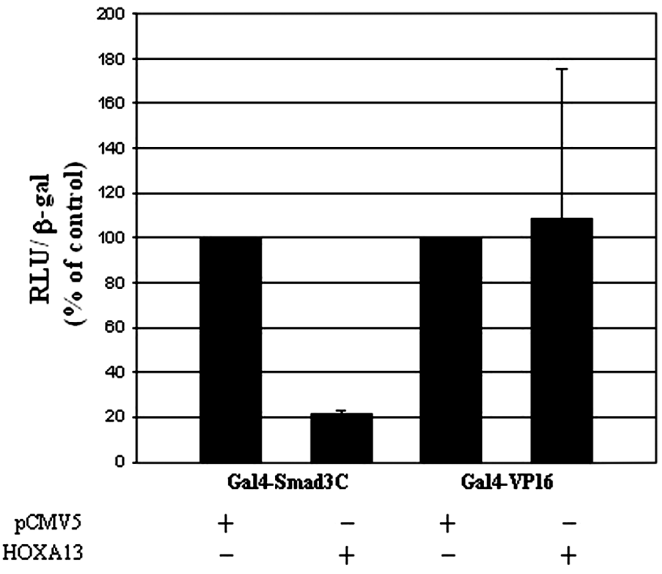


Figure 7. HOX proteins repress Smad3-MH2 domain-mediated transcriptional activation. Hep3B cells in 12 well plates were co-transfected with pG5B-Luc reporter construct (500 ng), GAL4-Smad3C (left) or GAL4-VP16 (right) fusion protein construct (10 ng) with or without HOXA13 construct. β-galactosidase (40 ng) vector was included to correct for transfection efficiency. Experiments were carried out as described in the legend to Figure 3, and luciferase activity in each sample was normalized to β-galactosidase activity; the average of two experiments is shown.

While it is theoretically possible that VP16 is strong enough to overcome repression by HOXA13, the data indicate that group 13 HOX proteins are capable of specifically inhibiting the transactivation functions of the Smad3 MH2 domain.

DISCUSSION

HOX/Smad interactions

To identify novel protein interactors of HOXA13, we created and screened a limb bud cDNA library using yeast two-hybrid analysis. We focused our attention on the specific interaction between HOXA13 and Smad5.

We showed that interaction of the Smad5 MH2 domain with paralog group 13 proteins was not a general property shared by the AbdB-like HOX proteins HOXA11 or HOXA9, although future exploration of the potential for interaction with HOX groups 10 and 12 proteins would test the hypothesis that Smad5 MH2 interaction is specific to group 13. We also mapped the interaction to the MH2 Smad domain, showed that Smad 1 and 2, but not Smad4, also interact with HOXA13 and demonstrated a negative effect of HOXA13 on TGF-β-stimulated promoter activation, Smad3/Smad4-activated promoter function and Smad3-C-terminus activation function. While the data indicate that one function of the HOXA13/Smad5 interaction may be to negatively modulate Smad transcriptional activation function, cellular and gene contexts may determine whether HOXA13/Smad5 interactions result in repression or activation.

Smad1, a BMP-regulated Smad, interacts with HOXC8 (41) disrupting HOX DNA binding, and thereby repression of gene expression (40). Two HOXC8 interaction domains were found in Smad1 (HID1 and HID2) that reside in the MH1 and linker regions. In our studies, HOXA13 did not interact with the homologous HID1/HID2 regions of Smad5 but rather with the C-terminus that contains the highly conserved MH2 domain. The MH2 domain of Smad proteins is well known for protein–protein interactions (23), yet this is the first report demonstrating an interaction between HOX proteins and the MH2 domain of Smad proteins. In Smad proteins, the MH1 domains bind to DNA and both MH1 and MH2 domains bind numerous proteins (24). The MH2 domain is capable of binding to transcriptional co-activators and is capable of activating transcription in heterologous contexts. However, Smad proteins may transcriptionally activate one promoter while repressing another in the same cell type depending on gene-specific co-factor complexes. Thus, one hypothesis is that HOX group 13 proteins repress Smad transcriptional activation by direct binding to the activation motif, presumably interfering with recruitment of co-activators or with Smad interaction with the basal transcription complex. Interestingly, Smad protein MH2 transactivation functions can occur through CBP/p300, which has also been shown to interact with HOX proteins (50). The prospect of a ternary complex of Smads/HOX/CBP at promoters seems plausible. Conceivably, HOX group 13 proteins could interfere with Smad/CBP interaction by binding to the MH2 domain with the Smad protein on or off of DNA. Perhaps HOXA13 binds to both proteins at promoters. Further studies are needed to determine the mechanism of HOXA13 repression of Smad-mediated activation and to define the Smad and HOX peptide sequences necessary for interaction. For example, do HOX proteins associate with R-Smad/Smad4 complexes after translocation to the nucleus or can association occur in the cytoplasm? Does association with HOX proteins pull R-Smads off of DNA or lead to R-Smad/Smad4 dissociation in solution?

HOXA13 and HOXD13 also interact with the MH2 domains of Smad1, another BMP-regulated Smad, Smad2, a mediator of TGF-β/Activin signaling, and interfere specifically with Smad3 C-terminal domain transactivation function. These results indicate that the functional interactions with Smads that are possible in tissues where group 13 HOX proteins are expressed are potentially quite broad. We showed that the group 13 HOX proteins were unable to interact with the common Smad heterodimerization partner, Smad4. This lack of interaction is consistent with the lower sequence identity to the Smad5 C-terminal region for Smad4 (46.3%) compared with Smad1 (97.5%), Smad2 (78.6%) and Smad3 (78.1%). Even though HOXA13 and HOXD13 were able to interact with Smad5 amino acids 175–465, more anterior *AbdB*-like HOX proteins HOXA9 and HOXA11 could not. This is distinctly different from HOXA9, which binds to Smad4 (48) but not to Smad5 (our study), Smad2 or Smad3 (48). We did not test a group 12 HOX protein; however, paralog-specific interactions on DNA have been described for other HOX co-factors including PBX (HOX proteins 1–10) and MEIS (HOX proteins 9–13) (16,19,51,52). It seems likely that the association of HOX proteins with Smads is very broad, which might be anticipated. Target gene regulation has been demonstrated through combinatorial interactions between HOX proteins and the TGF-β signaling pathway effector Mad (Smad ortholog) in *Drosophila* (53), suggesting the potential for conservation, expansion and evolution of selectivity of the

interactions as a result of gene and cluster duplication. Paralog-specific Smad interactions could be one way to modulate the *in vivo* functional specificity of Hox genes, and the potentially wide-ranging, yet selective, interactions may constitute a Hox/Smad code.

We also showed that monomeric DNA-binding capability, via experimental utilization of the HOXD13$^{IQN>AAA}$ mutant protein, is not necessary for the repression of TGF-β1- or Smad-stimulated promoter activity. Interestingly, Smad1 antagonizes the repressive effects of HOXC8 on the regulation of reporter vectors containing the *osteoprotegrin* and *osteopontin* promoters by displacing HOXC8 (40,41,54) and Smad4 interferes with the ability of HOXA9 or HOXC8 to bind DNA and repress the expression of the *osteopontin* promoter (48). In contrast, repression of Smad activation of gene expression by HOX group 13 proteins is mediated without HOX monomeric DNA-binding capability. A similar mechanism has been observed before for transcriptional activators whose physical binding and mutual antagonism leads to the repression of transcription and has been described for GR and AP1 (55), GR and NFk-B (56,57), GR and NFGI-B (58), Tpit and SF-1 (59), Pitx1 and IRF3 or IRF7 (60) and for the homeodomain-containing protein Pit1 and Gata2 (61). In the latter two cases, disruption of the ability of the homeodomain to bind to DNA did not interfere with binding to the other factors or transrepression. Our studies suggest that HOX and Smad proteins might reciprocally antagonize their ability to regulate gene expression. This may represent a more common *in vivo* theme whereby HOX proteins regulate downstream gene expression.

In what cellular contexts might HOXA13 and Smad5 function together?

Further work outside of the scope of this study is needed to determine the *in vivo* cellular context(s) for which HOX A13/D13/R-Smad interactions are important. The developing limb bud is a logical site to examine and a potential genetic interaction might be tested in crosses of appropriate Smad mouse mutants to those of *Hoxa13* and/or *Hoxd13*. BMP and TGF-β-signaling is important to several aspects of limb development, including outgrowth (62), cartilage formation and differentiation (63–66), digit identity (67), interdigital mesenchyme apoptosis (65,66,68) and cessation of outgrowth (69).

However, *Hoxa13* is also expressed in the caudal reproductive, genitourinary and digestive tracts, prostate, umbilical vasculature, allantois, migrating myoblasts and placenta (12,15,70,71), among others. Moreover, *Smad5* is also important for growth and differentiation of the vasculature of the early embryo, including vessels in the limbs (26) and it is highly expressed in the genitourinary tract (72). Importantly, critical sites of *Hoxa13* expression and function include the umbilical artery (UA) (12,15) where HOXA13 deficiency leads to umbilical artery stenosis and mutant fetal death at midgestation. Stadler *et al*. (12) showed that UA walls of *Hoxa13*$^{-/-}$ embryos fail to organize appropriate cellular boundaries between the endothelium and the mesenchyme. The loss of ephrin ligand and receptor expression in both the limbs and UA results was associated with a loss of cellular adhesion and subsequently to the failure to form proper cell boundaries. *Hoxa13* mutants also exhibit defective growth of the genital bud (9,13,15). Hypospadias may occur in humans with HFGS secondary to mutations in *HOXA13* (4), and in *Hoxa13* mutant mice hypospadias is associated with reduction of Bmp signaling (13). Capillary vessels in the developing glans penis or clitoris of mutant mice are abnormally enlarged, which are highly reminiscent of the vascular phenotypes of *Smad5*, *Bmp4*, *Tgf*β-1 and *Tgf*β receptor II knockout mice (26,72–75). *Hoxa13* loss, other than reducing Bmp signaling, may also alter Smad activity via deficient HOXA13/Smad protein interactions in these structures. HOXA13/Smad interaction may also be important in the proper development of the vasculature of the UA.

There is a growing list of co-factors that have been shown to interact with HOX proteins. For the *AbdB*-like proteins, genetic and physical interactions have recently been identified between Gli3 and HOXD12 in the limb bud (76), and *in vitro* interactions have been demonstrated between HOXD13 and both CBP and Gli3 (50,76). In this context, it is noteworthy that interactions have been reported between Smads 1, 2, 3 and 4 and C-terminally truncated forms of Gli3 (77). Thus, there appears to be the potential for higher order complexes of potentially different kinds involving GLI3/SMAD/HOX/CBP/MEIS/PBX proteins or, at the very least, a dynamic equilibrium *in vivo* that may modulate their respective activities in tissues where they are co-expressed. Finally, our data also adds further support to the role of HOX protein functions that do not require monomeric DNA-binding capability, and future work will be needed to more fully understand the scope of this mode of HOX function.

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